

Effects of Temperature and Glycerol and Methanol-Feeding Profiles on the Production of Recombinant Galactose Oxidase in *Pichia pastoris*

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Optimization of protein production from methanol-induced Pichia pastoris cultures is necessary to ensure high productivity rates and high yields of recombinant proteins. We investigated the effects of temperature and different linear or exponential methanol-feeding rates on the production of recombinant Fusarium graminearum galactose oxidase (EC 1.1.3.9) in a P. pastoris Mut⁺ strain, under regulation of the AOX1 promoter. We found that low exponential methanol feeding led to 1.5-fold higher volumetric productivity compared to high exponential feeding rates. The duration of glycerol feeding did not affect the subsequent product yield, but longer glycerol feeding led to higher initial biomass concentration, which would reduce the oxygen demand and generate less heat during induction. A linear and a low exponential feeding profile led to productivities in the same range, but the latter was characterized by intense fluctuations in the titers of galactose oxidase and total protein. An exponential feeding profile that has been adapted to the apparent biomass concentration results in more stable cultures, but the concentration of recombinant protein is in the same range as when constant methanol feeding is employed. © 2014 The Authors Biotechnology Progress published by Wiley Periodicals, Inc. on behalf of American Institute of Chemical Engineers Biotechnol. Prog., 000:000–000, 2014

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Introduction

Galactose 6-oxidase¹ (GalOx; D-galactose:oxygen 6-oxidoreductase; EC 1.1.3.9) is a monomeric free-radical copper oxidase produced and secreted by various fungal spe-

cies,² including species of the genus *Fusarium*.^{3,4} This enzyme catalyzes the oxidation of the C6 hydroxyl group of galactose, and also other primary alcohols to aldehydes, producing hydrogen peroxide.⁵ The reaction involves a transfer of two electrons, despite the single copper in the active site.⁶ Being among the simplest copper-containing oxidases, GalOx produced by the genus *Fusarium* –and especially *Fusarium graminearum*– has been studied extensively.^{5,7–9}

GalOx is currently used in several biotechnological applications, due to its selectivity and unique mode of action of producing a reactive aldehyde functionality and hydrogen peroxide. It has been employed, for example, as a possible dental anti-plaque system^{10,11} and in various analytical methods for the determination of lactose and other galactosides,^{12–14} in glycoprotein detection and bioconjugation,^{15,16} and in disease diagnostics.^{17,18} Likewise, GalOx has been used for chemo-enzymatic polysaccharide functionalization

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in the production of novel biopolymers and cellulosic materials.^{19–24} GalOx has also been the subject of significant protein engineering efforts to improve production and stability, and to diversify substrate specificity in order to expand the range of application of the enzyme.^{2,9,25–28}

The numerous industrial and medical applications of GalOx require scalable production strategies. Thus, this enzyme has been produced heterologously in large amounts in *Escherichia coli*^{2,26} and *Pichia pastoris*.²⁹ A more recent comparative study from our laboratories indicated that expression of GalOx-encoding genes in *P. pastoris* resulted in higher volumetric productivity in shaking-flask cultures than with common *E. coli* systems. Coupled with the ability to secrete protein directly into the medium, in the presence of the α -factor signal peptide and the simplicity of subsequent downstream processing, this suggests that the yeast system would be advantageous for scale-up of GalOx production.³⁰ In addition, *P. pastoris* remains a popular platform for the production of recombinant enzymes due to its high specific growth rate, its strong preference for respiratory growth compared to fermentative yeasts, its ability to grow on simple growth medium, and its convenience regarding the genetic manipulations required. Also, it has the ability to perform eukaryotic protein modifications, such as proteolytic processing, folding, disulfide bond formation and glycosylation, and exhibits high levels of intra- and extracellular protein expression compared to cell culture systems of higher eukaryotes.

One very efficient expression system of *P. pastoris* is regulated by the alcohol oxidase 1 (AOX1) promoter. The native alcohol oxidase is the first enzyme in the catabolic pathway of methanol, and it is strongly inducible by methanol but highly repressed by other carbon sources such as sugars or ethanol.³¹ Integration of a recombinant protein in the *P. pastoris* genome under the regulation of the AOX1 promoter can lead to high protein production.³² Over 600 recombinant proteins have been successfully produced heterologously in *P. pastoris*,³³ reaching high production yields commonly in the range of milligrams to grams per liter of culture. Methanol can be toxic at high concentrations, however, due to the accumulation of formaldehyde and hydrogen peroxide, both of which are products of the assimilation of methanol.³⁴ Thus, the methanol-feeding rate is of utmost importance for efficient protein production, and several different methods have been examined.^{35,36}

The methanol-feeding strategy generally suggested by the manufacturer of a widely-used *P. pastoris* expression system (Invitrogen Co., San Diego, CA, USA; “*Pichia* Fermentation Process Guidelines”) is based on monitoring of the methanol concentration with the dissolved oxygen tension (DOT) spike method. Methanol feeding is suspended regularly and the time required for the DOT to increase 10% is determined. If it is less than 1 min, methanol is considered the limiting factor and an increase in the feeding is proposed. During this process, the methanol fed is expected to be consumed instantly; its concentration in the cultivation broth is practically zero. This, however, may not be the optimal process design for the methanol fed-batch phase, and therefore alternative methanol-feeding strategies have been applied in the literature (i.e., providing methanol at constant, linear or exponential rates), which have indicated that constant feeding is the most efficient for recombinant protein production.³⁶ When constant feeding rates are applied, methanol is continuously diluted in the growth medium and the amount that is being fed to each cell decreases, as the biomass increases.³⁷

To our knowledge, the present work describes the first-ever attempt to optimize the recombinant *F. graminearum* GalOx production using a *P. pastoris* Mut⁺ SMD1168H strain under controlled fermentation conditions. Our aim was to evaluate the effect of different feeding profiles of glycerol and methanol, and also culture temperature, during the methanol-induction phase on the titre of active GalOx. Although this study describes a rather product-specific approach, our results provide insights into the level of tolerance of *P. pastoris* under these conditions, which will probably apply to similar production strategies employing SMD1168H and other Mut⁺ strains as well.

Materials and Methods

Strains

In this study we used the recombinant strain PCE of *P. pastoris*, which overproduces the galactose oxidase of *Fusarium sp.* that has been His-tagged at the C-terminus.³⁰ It is derived from the SMD1168H strain, which is deficient in protease activity,³⁸ and it was maintained at -80°C in 20 % glycerol.

Growth media

The composition of the fed-batch cultivation medium for *P. pastoris* was as follows (per L): 40 g glycerol, 0.93 g CaSO₄, 18.2 g K₂SO₄, 14.9 g MgSO₄·7H₂O, 4.13 g KOH, 7 g K₂HPO₄, 22.7 mL H₃PO₄ (85 %), 0.01 % v/v Breox FMT30 antifoam, and 12 mL of trace-elements solution. The composition of the trace-elements solution was (per L): 6 g CuSO₄·5H₂O, 0.08 g NaI, 3 g MnSO₄·H₂O, 0.2 g Na₂MoO₄·2H₂O, 0.02 g H₃BO₃, 0.5 g CoCl₂, 20 g ZnCl₂, 65 g FeSO₄·7H₂O and 0.2 g biotin. NH₄OH (28 % v/v) was used as a nitrogen source and for pH adjustment.³⁹

Precultures were prepared in Buffered Glycerol Complex Medium (BMGY) in 500-mL shake flasks, with the following composition (per L): 10 g glycerol, 10 g yeast extract, 20 g peptone, 1.34 % (v/v) yeast nitrogen base (YNB), and 0.004 g biotin in 100 mM potassium phosphate buffer, pH 6.0.

Preparation of inoculum

A fresh culture with the recombinant *P. pastoris* PCE strain was grown for 18 to 20 h at 30°C and 250 rpm in 500-mL baffled flasks containing 50 mL of BMGY. The cells were centrifuged and resuspended in fresh BMGY medium to a final optical density (OD) of approximately 0.3 for inoculation of bioreactors.

Cultivation conditions

To determine the physiological characteristics of the strain and enhance extracellular galactose oxidase production, fed-batch fermentations were performed, using 2.7 L DasGip bioreactors (DasGip AG, Jülich, Germany) equipped with two Rushton four-blade disc turbines and pH and temperature control. Temperature was maintained at 25 or 30°C and pH was maintained at 6.0 by automatic addition of NH₄OH, and both were kept constant throughout the cultivation. The initial stirring speed was set to 1,000 rpm and the aeration rate to 1.5 vvm; these were both controlled automatically to prevent conditions of oxygen limitation conditions, by restricting DOT to a minimum of 20 %.

The fermentations were initiated with a batch phase with 40 g L⁻¹ glycerol as the sole carbon source. After its

exhaustion, a high concentration (50 % v/v) of glycerol feed was applied, at a rate of $36 \text{ mL h}^{-1} L_{\text{initial culture}}^{-1}$ for different time periods. When the glycerol concentration became the limiting factor for growth, determined by the increase in DOT, the methanol-feeding phase was initiated. Methanol was provided at a rate of 3 mL h^{-1} for adaptation. When DOT became stable, the cells were adapted to methanol as the sole carbon source, and different methanol-feeding rates were used for the production phase.

Quantification of biomass, enzymatic activity, and protein

Cell dry weight was determined using nitrocellulose filters (pore size $0.45 \mu\text{m}$; Gelman Sciences) from weight difference before and after biomass filtration, after drying in an oven at 100°C for 24 h. GalOx was activated with the addition of CuSO_4 as previously described,³⁰ and its activity was assayed by using a spectrophotometric method with 2,2'-azino-bis-(3-benzthiazoline-6-sulfonic acid) (ABTS) as substrate.⁵ The enzyme-substrate reaction produced a soluble blue-green product that was measurable at 405 nm (Southern Biotech, Birmingham, AL, USA). Protein was quantified using the Bio-Rad protein assay with bovine serum albumin as standard (Bio-Rad Laboratories).

Results and Discussion

To optimize the production of recombinant GalOx in a *P. pastoris* Mut⁺ strain, the effects of different induction temperatures and of various methanol- and glycerol- feeding conditions were investigated.

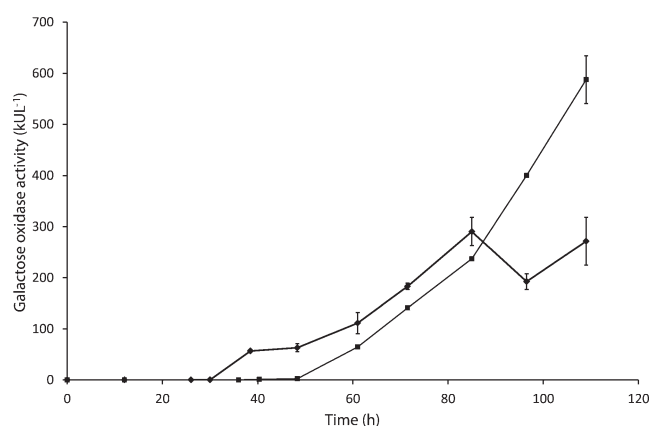


Figure 1. Volumetric GalOx activity in the fermentation broth at 25°C (■) and 30°C (◆). Time corresponds to the total time of cultivation. Methanol feeding was initiated at 30 h. The detectable production of active and correctly folded GalOx sets off earlier at 30°C , apparently due to the higher biomass concentration (data not shown). It stabilizes after 85 h of culture. At 25°C , the volumetric GalOx activity increases continuously to 600 kU L^{-1} .

Effect of temperature

There have been several reports on the effect of induction temperature on recombinant protein production by *P. pastoris*. It is generally suggested that a decrease in temperature to below 30°C has a positive effect on protein production.^{40–42} More specifically, Batra et al. observed a 2.5 to 10-fold increase in the yield of a recombinant protein when the induction phase was sustained at 20°C rather than 30°C .⁴² Also, Dragosits et al. reported an increase in the yield of the antibody fragment Fab 3H6 from 5.4 mg L^{-1} to double and triple values, and an increase in the specific productivity from 21 to 45 and $65 \mu\text{g Fab g}^{-1}_{\text{cell biomass}} \text{ h}^{-1}$ when the temperature was reduced from 30 to 25 and 20°C , respectively, in order to achieve a specific growth rate (μ) of 80 % and 60 % of μ_{max} in chemostat experiments.⁴¹

In the present study, two different temperatures were used for both the growth phase and the induction phase (25°C and 30°C). The detectable total protein production occurred sooner when *P. pastoris* was grown at 30°C (most probably due to a higher biomass concentration), but the volumetric GalOx productivity and the biomass concentration became rather constant after 85 h (Figure 1). We believe that after 85 h, carbon limitation occurred due to the higher temperature, under which the cells grow faster, and due to the applied constant methanol feeding profile. On the other hand, at 25°C the volumetric GalOx activity in the fermentation broth continued to increase and reached 600 kU L^{-1} after 110 h, when the cultivation was terminated. Also, the maximum specific activity was almost doubled at 25°C (Table 1), indicating that the production of correctly folded recombinant protein was favored at this temperature relative to 30°C . Although the total extracellular protein concentration was higher at 30°C , the volumetric productivity of active GalOx was 66 % higher when the production was performed at 25°C (Table 1). Again, this agrees with a previous report that at 30°C the enzyme produced can be partially misfolded and therefore more inactive than when produced at the lower temperature.³⁰

High vs. low exponential methanol-feeding profiles

After the glycerol batch and fed-batch phase was over and glycerol became the limiting factor for growth, methanol was provided in the culture medium at a rate of $3 \text{ mL h}^{-1} L_{\text{initial culture}}^{-1}$. When the DOT level in the bioreactor stabilized, the cells were regarded as being adapted to methanol. To optimize the volumetric productivity of active enzyme, which is still the key target parameter for bioprocess engineers, different exponential methanol-feeding profiles were tested at 25°C (Table 2). We designed our experiments in order to determine the most efficient way of avoiding methanol intoxication in the fermentation broth. Thus, there would be no need to follow the fermentation with a methanol detector, which can be affected by other compounds such as ammonia. Furthermore, accumulation of methanol negatively affects the

Table 1. Galactose Oxidase Activity and Productivity at Different Induction Temperatures

| Fermentation | Protein concentration (g L^{-1}) | Volumetric activity (kU L^{-1}) | Specific activity (U mg^{-1}) | Volumetric productivity ($\text{kU L}^{-1} \text{ h}^{-1}$) |
|--|---|--|--|---|
| Constant methanol feed ($3 \text{ mL h}^{-1} L_{\text{initial culture volume}}$) at 25°C | 0.76 | 588 | 768 | 5.39 |
| Constant methanol feed ($3 \text{ mL h}^{-1} L_{\text{initial culture volume}}$) at 30°C | 0.93 | 354 | 396 | 3.24 |

Table 2. Methanol-Feeding Profiles, Total Protein Concentration, and GalOx Productivity at Low and High Exponential Feeding Profiles

| Experiment | Methanol feeding profile* | | Protein concentration (g L ⁻¹) | Volumetric activity (kU L ⁻¹) | Specific activity (U mg ⁻¹) | Volumetric productivity (kU L ⁻¹ h ⁻¹) |
|------------|------------------------------------|------------------|--|---|---|---|
| A | $F(t) = e^{0.28t}$ | High exponential | 0.45 | 66.4 | 148 | 1.3 |
| B | $F(t) = e^{0.28\text{EXP}(0.28t)}$ | High exponential | 0.35 | 46.1 | 132 | 1.4 |
| C | $F(t) = 6e^{0.1t}$ | Low exponential | 0.8 | 501.3 | 627 | 9.1 |
| D | $F(t) = 4.76e^{0.1t}$ | Low exponential | 0.97 | 564.5 | 582 | 10.3 |

Low feeding profiles resulted in higher total GalOx activity and also higher volumetric productivity.

*F(t) corresponds to the feeding flow rate in mL h⁻¹ and t refers to the time in h after the start of the induction phase.

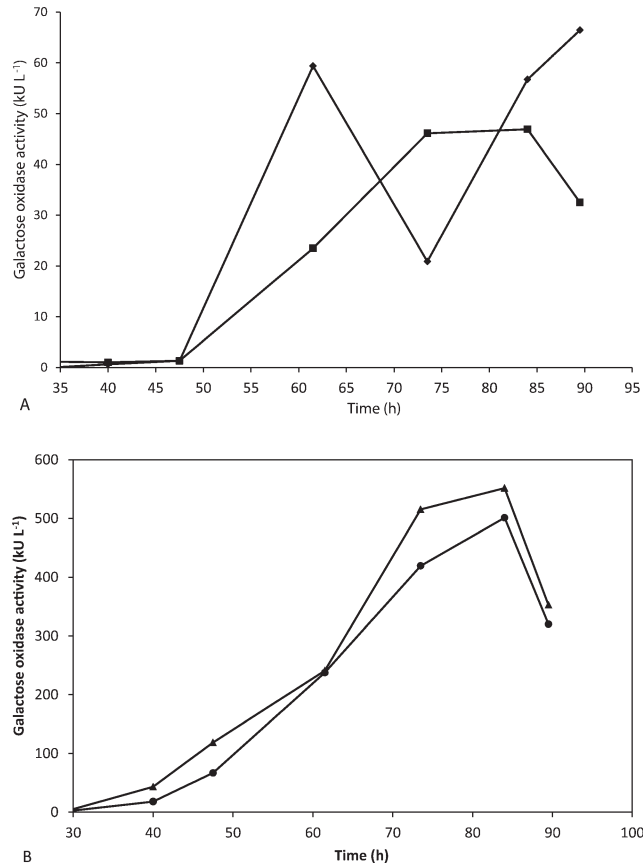


Figure 2. Volumetric GalOx activity as a function of time in culture using (A) high methanol-feeding rates, following the function $F(t) = e^{0.28t}$ (◆) and $F(t) = e^{0.28\text{EXP}(0.28t)}$ (■), and (B) low methanol-feeding rates following the function $F(t) = 6e^{0.1t}$ (●) and $F(t) = 4.76e^{0.1t}$ (▲). Under mild conditions, the volumetric GalOx activity in the fermentation broth was almost 10 times higher than in the former case. Time refers to the total time in culture. The exponential phase started at 40 h.

viability of the cells and it also warrants safety considerations, especially in large-scale production processes.

The first two experiments (experiments A and B; Table 2) were performed with high exponential feeding rates (Figure 2A): experiment A at feeding rate that was double the maximum specific growth rate of *P. pastoris* on methanol which was reported to be 0.14 h⁻¹,⁴³ and experiment B with a double exponential feeding rate with the purpose to increase the stress for the cells. The volumetric productivity was basically the same in these two experiments, i.e., 1.3 and 1.4 kU L⁻¹ h⁻¹, respectively. However, production of active GalOx was found to be 148 U mg⁻¹ in fermentation A, where a lower feeding profile was used, and 132 U mg⁻¹ in fermentation B. These

two experiments already indicated that there was a positive effect of a lower methanol-feeding rate on the production of active GalOx. To confirm this hypothesis, we performed two similar fed-batch experiments with only small differences in the initial flow and the same exponential feeding profiles, which were lower than the maximum specific growth rate (experiments C and D; Table 2). In fact, the volumetric productivity was almost 8-fold higher when low methanol-feeding rates were applied rather than high feeding rates. Not only was the overall amount of extracellular protein significantly higher, but also the amount of active, correctly folded GalOx increased almost 5-fold, as shown from the values of the specific GalOx activity (Table 2). The small differences in the initial flow did not seem to affect the yield, although experiment D, with the lower initial flow, resulted in slightly better volumetric productivity and volumetric activity.

Optimization of the methanol-feeding profile

Considering that low exponential methanol-feeding rates proved to be favorable in terms of GalOx productivity, the effect of different low exponential feeding rates was further evaluated in order to fine-tune the feeding profile (Table 3). For this purpose, the conclusions from the model developed by Zhang et al.³⁷ were used to correlate the specific growth rate (μ), the product formation, and the methanol concentration. According to this model and their subsequent experimental results, the maximum μ was 0.08 h⁻¹ and 0.0709 h⁻¹ respectively, when the methanol concentration in the growth medium was kept constant at 3.65 g L⁻¹, while lower or higher concentrations resulted in methanol limitation or inhibition. In this respect, we designed four different fed-batch experiments, where the feeding profiles were designed in such a way as to obtain certain specific growth rates, as shown in Table 3. We wanted to cover different μ -ranges, from the predetermined maximum of around 0.0709 h⁻¹ to only a third of that. The initial flow (α , Table 3) was adjusted, so that the range of the pumps would fit the range of flows required in the duration of the culture.

As shown in Table 3, the highest volumetric productivity of 16 kU L⁻¹ h⁻¹ was obtained at a medium specific growth rate of 0.05 h⁻¹ (experiment F), compared to the 14 kU L⁻¹ h⁻¹ at a low specific growth rate and 9.9 kU L⁻¹ h⁻¹ at a high specific growth rate. Volumetric activity and protein concentration were similarly higher at the medium specific growth rate. However, the specific activity of extracellular GalOx in the fermentation broth was higher at a lower specific growth rate of 0.02 h⁻¹ (experiment G). At the lower specific growth rate, more correctly folded and active GalOx was apparently secreted into the fermentation broth than at higher specific growth rates.

In order to investigate the potentially toxic effect of methanol, even at the low specific growth rate, we performed

Table 3. Methanol-Feeding Rate, Exponential Feeding Rate in Relation to the Optimum μ_{\max} According to Zhang et al.,³⁷ Protein Concentration, and GalOx Productivity in Experiments E-H

| Experiment | Exponential growth rate | Methanol feeding profile* $F(t) = \alpha e^{\beta t}$ | | Exponential feeding rate relative to optimum μ_{\max} (%) [†] | Protein concentration (g L ⁻¹) | Volumetric activity (kU L ⁻¹) | Specific activity (U mg ⁻¹) | Volumetric productivity (kU L ⁻¹ h ⁻¹) |
|------------|---|--|---------|--|--|---|---|---|
| | | α | β | | | | | |
| E | High | 26.96 | 0.071 | 100 | 0.57 | 346 | 608 | 9.9 |
| F | Medium | 20.89 | 0.0497 | 70 | 0.81 | 559 | 694 | 16.0 |
| G | Low | 9.64 | 0.0213 | 30 | 0.61 | 491 | 811 | 14.0 |
| H | Low with low initial methanol concentration | 2.22 | 0.0213 | 30 | 0.38 | 239 | 630 | 10.7 |

*F(t) corresponds to the feeding flow rate in mL h⁻¹ and t refers to the time in h after the start of the induction phase.

[†]Predicted optimum μ_{\max} , according to the results by Zhang et al.,³⁷ is 0.071 h⁻¹, when methanol concentration in the medium remains constant at 3.65 g L⁻¹.

Table 4. Experimental Design to Investigate Any Possible Influence of the Glycerol-Feeding Phase and Different Methanol-Feeding Profiles on the Production of Active GalOx

| Experiment | Glycerol feeding phase (h) | Methanol feeding profile* | Protein concentration (g L ⁻¹) | Volumetric activity (kU L ⁻¹) | Specific activity (U mg ⁻¹) | Volumetric productivity (kU L ⁻¹ h ⁻¹) |
|----------------|----------------------------|--|--|---|---|---|
| I | 4 | 3 mL h ⁻¹ L _{initial culture volume} | 1.38 | 1447 | 1045 | 10.56 |
| J | 8 | 3 mL h ⁻¹ L _{initial culture volume} | 1.12 | 1508 | 1343 | 11.01 |
| K [†] | 8 | $F(t) = 3.6 e^{0.0213t}$ | 0.9 | 909 | 1005 | 6.63 |
| | | | 1.61 (131 h) | 2016 (131 h) | 1250 (131 h) | 15.39 (131 h) |
| L | 8 | constant according to wet cell weight | 1.05 | 1588 | 1509 | 11.59 |

The protein concentration, volumetric activity, specific activity, and volumetric productivity at the final time point (137 h) are presented.

*F(t) corresponds to the feeding flow rate in mL h⁻¹ and t refers to the time in h after the start of the induction phase.

[†]For experiment K, the respective values at 131 h are also shown, to demonstrate their fluctuation.

experiment H (Table 3), where the methanol-feeding was provided at the same exponential rate but with a lower starting concentration. As can be seen from Table 3, the lower initial methanol concentration did not result in the same volumetric productivity, but in a 25% reduction (14.0 and 10.7 kU L⁻¹h⁻¹, respectively). Not only was less total protein secreted by the cells, but also less active GalOx was produced, as seen by the reduced specific activity (Table 3). It appears that induction efficiency was restricted under these conditions by the low methanol concentration and also by the slightly lower biomass concentration. Indeed, the latter approximated 82 g L⁻¹ at medium specific growth rate, but was lower in all other cases.

Influence of the glycerol-feeding phase on the methanol-induction phase

Considering that higher biomass concentrations have been reported,⁴⁴ pinpointing the relationship between recombinant protein productivity and biomass concentration at the start of the induction phase, we performed four additional fed-batch experiments to investigate a possible influence of the glycerol fed-batch phase, and thus the biomass concentration, on the subsequent methanol-induction phase and the concomitant production of recombinant GalOx, and to further determine whether constant methanol feeding is more favorable for the production of active GalOx than an exponential feeding profile (experiments I-L, Table 4).

After 140 h of cultivation there was no difference in the volumetric productivity between experiment I (4 h of glycerol feeding) and J (8 h of glycerol feeding) (Figure 3). Of course, the production of active GalOx started sooner when glycerol was only fed in for 4 h, but already after around 80 h the amount of

active GalOx in the fermentation broth in experiments I and J were the same. Also, the cell concentration seemed to reach its maximum density at the end of the induction phase, yielding comparable final total protein titres. There is apparently no significant effect of the glycerol fed-batch phase on the subsequent production of active GalOx in the induction phase. Due to the fact that yeasts consume more oxygen and produce more heat when metabolizing methanol rather than glycerol,⁴³ a prolonged glycerol-feeding phase should be favorable. To investigate this, we used a glycerol-feeding phase of 8 h in the remaining fed-batch experiments (K and L; Table 4) where we wanted to test whether an exponential feeding rate with a predefined feeding function gives a higher titre of product than an exponential feeding rate, which we adjusted according to the current wet cell weight. The latter strategy involving an exponential feeding rate is where the cells are fed at a constant specific substrate uptake rate (q_s), which is similar to that of recently published studies.^{45,46} As shown in Figure 3, the methanol-feeding strategy based on constant q_s was superior to an exponential feeding profile with a predefined feeding function regarding volumetric productivity. Furthermore, the latter feeding profile resulted in strong fluctuations in the production of active GalOx, especially in the later phases of the induction period (Figure 3). Also, the total extracellular protein fluctuated similarly (data not shown), which suggests that methanol becomes inhibitory at certain concentrations and cell densities. By applying an exponential feeding profile with a predefined feeding function, the culture apparently cannot be controlled reliably: potential changes in yields cannot be predicted, there is a risk of over-feeding or starvation periods for the cells, and the uncontrolled conditions could result in the production of undesirable metabolites. Even so, although exponential methanol-feeding rates appear to be

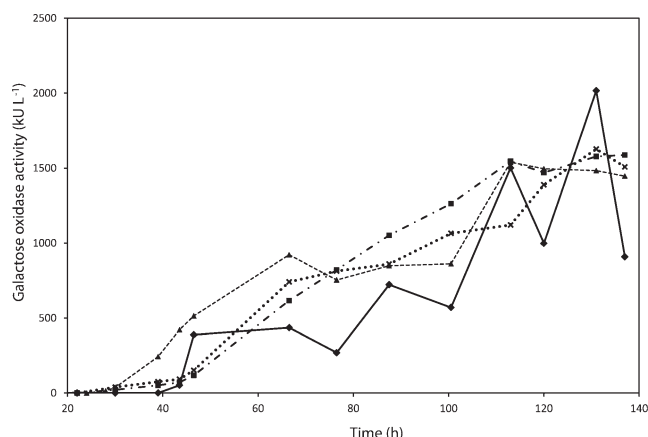


Figure 3. Volumetric GalOx activity in the fermentation broth under different fed-batch conditions; (I) 4-h glycerol-feeding phase and constant methanol-feeding rate ($3 \text{ mL h}^{-1} \text{ L}_{\text{initial culture volume}}^{-1}$) (\blacktriangle), (J) 8-h glycerol feeding phase with constant methanol feeding rate ($3 \text{ mL h}^{-1} \text{ L}_{\text{initial culture volume}}^{-1}$) (\times), (K) 8-h glycerol-feeding phase and exponential methanol-feeding rate [$F(t) = 3.6e^{0.0213t}$] (\blacklozenge), (L) 8-h glycerol feeding phase with constant methanol feeding rate to wet cell weight ratio (\blacksquare). Time corresponds to the total time of the cultivation. Methanol adaptation started at 30 h and lasted for 2 h.

detrimental for the productivity, during prolonged cultivation times the population manages to adapt to the high methanol concentration.

Despite the difference in the initial biomass concentration between the 4 h and the 8 h glycerol-feeding phases, the final active GalOx productivity was similar. This result is surprising and contradicts the report of Wang et al.,⁴⁴ who also examined the growth and protein production of a recombinant *P. pastoris* Mut⁺ strain. Although they found that the cell concentration reached similar final levels (122 g L^{-1}) regardless of its value at the start of the induction stage (62.5 , 90 or 122 g L^{-1}), at the end of the induction the enzyme titre was higher, proportionally to the initial biomass concentration (102 , 168 and 207 U mL^{-1} respectively), with similarly affected volumetric productivities (1.14 , 1.87 and $2.39 \text{ U mL}^{-1} \text{ h}^{-1}$). They also tested different constant methanol-feeding rates, following the highest biomass levels they achieved, and showed that in agreement (to some extent) with our results, the high methanol-feeding rates stress the cell machinery and negatively affect the process performance. In a relevant study, Cunha et al. demonstrated a positive effect of the initial biomass on the protein production during the induction phase.⁴⁷

Several studies to date have concentrated on the feeding strategies with glycerol and methanol regarding recombinant protein production in *P. pastoris*. Zhou et al. and Trinh et al., for example, investigated the effects of co-feeding methanol and glycerol during the induction phase. Zhou et al. reported a higher protein yield when co-feeding with glycerol and methanol.⁴⁸ Trinh et al. investigated the effect of three different strategies on protein production, in addition to the co-feeding. They reported that feeding with methanol at a predefined exponential rate to a μ of 0.02 h^{-1} was superior for endostatin production to cultivations controlled by a methanol sensor or controlled by the oxygen consumption, in terms of specific productivity ($0.72 \text{ mg endostatin g}^{-1} \text{ DCW}$ compared to $0.32 \text{ mg endostatin g}^{-1} \text{ DCW}$ in both other cases).³⁵ On the other hand, Li et al. reported higher produc-

tivity when methanol was provided at a constant rate of 3 , 6 , or 10 g L^{-1} than with exponential feeding profiles.³⁶ Results are apparently inconsistent and thus recommendations are different. In our study, we tested and compared both feeding strategies (experiments J and K; Table 4). As shown in Figure 3, both feeding strategies basically resulted in the same range of final volumetric productivities, although the exponential feeding profile resulted in higher fluctuations in GalOx activity. These fluctuations were also evident in the protein concentrations (data not shown), indicating the uncontrollable reaction of the population to cycles of cell intoxication due to over-feeding and subsequent adaptation.

Conclusion

In this study, we tested different induction temperatures and different glycerol- and methanol-feeding strategies to optimize the production of GalOx with a recombinant *P. pastoris* Mut⁺ strain. Our results indicate that an induction temperature of 25°C is superior to 30°C , as both the production of active enzyme and the volumetric productivity increased up to 8-fold. Also, a low exponential methanol-feeding profile gives more active GalOx and almost 1.5-fold higher volumetric productivity than a high exponential feeding strategy. Although these findings correspond to previous reports in literature, they still might be product or strain-specific. The duration of the glycerol-feeding phase has no direct effect on the subsequent methanol-induction phase for the investigated Mut⁺ strain. In terms of oxygen consumption and heat production, we would recommend prolonged glycerol fed-batch phases leading to a higher initial biomass concentration for induction. A linear methanol-feeding profile and an exponential methanol-feeding profile resulted in basically the same volumetric productivities, while one adapted to the apparent biomass concentration in the bioreactor gives more stable cultures. By applying this method, the risk of under-feeding or over-feeding the cultures is omitted (e.g., when yields change during cultivation due to recombinant protein production). This finding is not product-specific and can be of great relevance for other recombinant *P. pastoris* Mut⁺ strains.

To our knowledge this is the first time that it is highlighted that despite the differences in the pre-induction biomass concentrations and the non-toxic methanol feeding profiles, there is a plateau in the volumetric activity values where all processes converge. A conservative methanol-feeding profile would actually contribute to the stability of the culture and avoid uncontrollable fluctuations in the concentrations of recombinant proteins.

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